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Dated 29 February 2000.

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PATENTS ACT 1953

334386

PROVISIONAL SPECIFICATION

DIAGNOSIS AND THERAPY OF PREMATURE OVARIAN FAILURE

We, **AUCKLAND UNISERVICES LIMITED**, a New Zealand company of UniServices House, 58 Symonds Street, Auckland, New Zealand, do hereby declare this invention to be described in the following statement:

(followed by page 1a)

In still a further embodiment, the presence or absence of the mutation is detected through analysis of the amino acid sequence of the expressed inhibin protein.

As a separate embodiment, the invention provides a method of prophylaxis and/or therapeutic treatment against POF of an individual identified as having a risk of POF by a method defined above or suspected to have such a risk which comprises the step of increasing, maintaining and/or restoring the active concentration of wild-type inhibin protein within said individual.

Conveniently, the method will be a gene therapy method and will involve supplying the individual with wild-type inhibin gene function.

Most preferably, the method will involve administering wild-type inhibin to the individual.

In still a further aspect, the invention provides for the use of inhibin in the manufacture of a medicament for treating or preventing POF.

DESCRIPTION OF THE INVENTION

As defined above, the method of the invention detects a predisposition to POF or is diagnostic of POF. The critical finding made by the applicants is that at least some instances of POF are due to an alteration (mutation) in the gene encoding inhibin. This finding forms the basis of the present invention.

Inhibin is structurally related to the TGF-β superfamily. The mature inhibin is a 31-32kDa heterodimeric glycoprotein consisting of an 18kDa α-subunit linked by two disulphide bonds to one of two 14kDa β-subunits (Halvorson and Decherney 1996). Therefore there are two forms of inhibin: inhibin A (α-β_A), and inhibin B (α-β_B). The homodimer of the β-subunit form the glycoprotein activin, which has an opposing function to inhibin. The inhibin subunits are encoded by three separate genes: INHα, INHβA, and INHβB.

As indicated above, INHα, INHβA, and INHβB, together with their non-coding flanking sequences and regulatory elements are collectively referred to herein as the "gene encoding inhibin".

Serum inhibin levels vary across the menstrual cycle and across each of the reproductive stages of life, suggesting that it is an important modulator of the pituitary-gonadal axis and gonadal function (Halvorson and Decherney 1996). Inhibin increases with the onset of puberty when ovarian function begins, shows cyclic function in the reproductive ovary, and decreases approaching menopause when the ovary enters the non-reproductive state.

The main function of inhibin in the female is the regulation of pituitary FSH secretion. Granulosa cells in both the developing follicle and the corpus luteum secrete inhibin in response to gonadotrophins and other factors such as IGF-1, TGF-β and activin. FSH-induced secretion is in-turn suppressed by EGF, TGF-α, and follistatin. Follistatin is an inhibin-like FSH suppressor which also acts as a binding protein for both inhibin and activin regulating their paracrine and autocrine functions (Halvorson and Decherney 1996).

Developing follicles appear to secrete inhibin B to suppress FSH secretion in the follicular phase. In the luteal phase the sole dimer secreted to regulate FSH is inhibin A. Inhibin along with it's regulation of FSH displays both autocrine and paracrine effects within the ovary. These include the regulation of steroidogenesis, cell growth, and cell differentiation. Inhibin increases androgen production by increasing theca cell responsiveness to LH. It has also been suggested that inhibin along with activin may be involved in a mechanism to select the dominant follicle and to prevent premature luteinisation (Wallace and Healy 1996).

However, to date, there has been no suggestion that an alteration/mutation in the gene encoding inhibin is in any way predictive of susceptibility to POF or is diagnostic of POF.

The amino acid and cDNA nucleotide sequences encoding inhibin are accessible from Genbank. Any change in either sequence is included in the scope of the term "mutation" as used herein.

In terms of inhibin non-coding flanking sequences and regulatory elements, mutations in these may cause transcript instability and/or transcriptional repression. Relevant regulatory regions include the sites for transcript splicing.

In one approach according to the present invention, alteration of the wild-type inhibin gene is detected.

"Alteration of a wild-type inhibin gene" encompasses all forms of mutations including deletions, insertions, missense and point mutations in the coding and noncoding regions. Deletions may be of the entire gene or only a portion of the gene. Point mutations may result in stop codons, frameshift mutations or amino acid substitutions.

Mutations leading to non-functional gene products are believed to primarily lead to POF. However, mutations which lead to decreased expression of the inhibin gene product may also lead to POF. Point mutation events may occur in regulatory regions, such as in the promoter of the gene, leading to loss or diminution of expression of the mRNA. Point mutations may also abolish proper RNA processing, leading to loss of expression of the inhibin gene product, or a decrease in mRNA stability or translation efficiency.

A preliminary analysis to detect deletions in DNA sequences can be performed by looking at a series of Southern blots of DNA cut with one or more restriction enzymes, preferably a large number of restriction enzymes. Each blot contains DNA from a series of normal individuals and from a series of test cases. Southern blots displaying hybridizing fragments (differing in length from control DNA when probed with sequences near or including the inhibin locus) indicate a possible mutation. If restriction enzymes which produce very large restriction fragments are used, then pulsed field gel electrophoresis ("PFGE") can be employed.

Detection of point mutations may be accomplished by molecular cloning of the inhibin gene and sequencing that gene using techniques well known in the art. Alternatively, the gene sequences can be amplified, using known polynucleotide amplification techniques, directly from a genomic DNA preparation from the sample tissue. The amplification techniques which can be used include methods such as the polymerase chain reaction (PCR), ligation amplification (or ligase chain reaction, LCR) and amplification methods based on the use of Q-beta replicase. These methods are well known and widely practised in the art. See, eg., US Patents 4,683,195 and 4,683,202 and Innis *et al.*, 1990 (for PCR); and Wu *et al.*, 1989a (for LCR). Reagents and hardware for conducting amplification are commercially available. Primers useful to amplify sequences from the inhibin region are preferably complementary to, and hybridize specifically to sequences in the inhibin region or in regions that flank a target region therein.

Inhibin sequences generated by amplification may be sequenced directly. Alternatively, but less desirably, the amplified sequence(s) may be cloned prior to sequence analysis. A method for the direct cloning and sequence analysis of enzymatically amplified genomic segments has been described by Scharf, 1986.

There are numerous well known methods for confirming the presence of a mutant gene. These include: 1) single stranded conformation polymorphism ("SSCP") (Orita *et al.*, 1989); 2) denaturing gradient gel electrophoresis ("DGGE") (Wartell *et al.*, 1990; Sheffield *et al.*, 1989); 3) RNase protection assays (Finkelstein *et al.*, 1990; Kinsler *et al.*, 1991); 4) allele-specific oligonucleotides (ASO's) (Conner *et al.*, 1983); 5) the use of proteins which recognize nucleotide mismatches, such as the *E. coli* mutS protein (Modrich, 1991); and 6) allele-specific PCR (Rano & Kidd, 1989). For allele-specific PCR, primers are used which hybridize at their 3' ends to a particular inhibin mutation. If the particular inhibin mutation is not present, an amplification product is not observed.

Other approaches which can also be used include the Amplification Refractory Mutation System (ARMS), as disclosed in European Patent Application Publication No. 0332435 and in Newton *et al.*, 1989. Insertions and deletions of genes can also be detected by cloning, sequencing and amplification. In addition, restriction fragment length polymorphism (RFLP) probes for the gene or surrounding marker genes can be used to detect alteration of an allele or an insertion in a polymorphic fragment. Such a method is particularly useful for screening relatives of an affected individual for the presence of the inhibin mutation found in that individual.

In the first three methods (ie., SSCP, DGGE and RNase protection assay), a new electrophoretic band appears. SSCP detects a band which migrates differentially because the sequence change causes a difference in single-strand, intramolecular base pairing. DGGE detects differences in migration rates of mutant sequences compared to wild-type sequences, using a denaturing gradient gel. RNase protection involves cleavage of the mutant polynucleotide into two or more smaller fragments. In an allele-specific oligonucleotide assay, an oligonucleotide is designed which detects a specific sequence, and the assay is performed by detecting the presence or absence of a hybridization signal. In the mutS assay, the protein binds only to sequences that contain a nucleotide mismatch in a heteroduplex between mutant and wild-type sequences.

Mismatches are hybridized nucleic acid duplexes in which the two strands are not 100% complementary. Lack of total homology may be due to deletions, insertions, inversions or substitutions. Mismatch detection can be used to detect point mutations in the gene or its mRNA product. While these techniques are less sensitive than sequencing, they are simpler to perform on a large number of samples.

An example of a mismatch cleavage technique is the RNase protection method. This method involves the use of a labeled riboprobe which is complementary to the human wild-type inhibin gene coding sequence. The riboprobe and either mRNA or DNA isolated from the test tissue are annealed (hybridized) together and subsequently digested with the enzyme RNase A which is able to detect some mismatches in a duplex RNA structure. If a mismatch is detected by RNase A, it cleaves at the site of the mismatch. Thus, when the annealed RNA preparation is separated on an electrophoretic gel matrix, if a mismatch has been detected and cleaved by RNase A, an RNA product will be seen which is smaller than the full length duplex RNA for the riboprobe and the mRNA or DNA.

The riboprobe need not be the full length of the inhibin mRNA or gene but can be a segment of either. If the riboprobe comprises only a segment of the inhibin mRNA or gene, it will be desirable to use a number of these probes to screen the whole mRNA sequence for mismatches.

In similar fashion, DNA probes can be used to detect mismatches, through enzymatic or chemical cleavage. See, eg., Cotton *et al.*, 1989; Shenk *et al.*, 1975; Novack *et al.*,, 1986. Alternatively, mismatches can be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes. See eg. Cariello, 1988. With either riboprobes or DNA probes, the cellular mRNA or DNA which might contain a mutation can be amplified using PCR before hybridization. Changes in DNA of the inhibin gene can also be detected using Southern hybridization, especially if the changes are gross rearrangements, such as deletions and insertions.

DNA sequences of the inhibin gene which have been amplified by use of PCR may also be screened using allele-specific probes. These probes are nucleic acid oligomers, each of which contains a region of the inhibin gene sequence harboring a known mutation. For example, one oligomer may be about 20 nucleotides in length, corresponding to a portion of the inhibin gene sequence. By use of a battery of such allele-specific probes, PCR amplification products can be screened to identify the presence of a previously identified mutation in the inhibin gene.

Hybridization of allele-specific probes with amplified inhibin sequences can be performed, for example, on a nylon filter such as Hybond. Hybridization to a particular probe under stringent hybridization conditions indicates the presence of the same mutation in the sample as in the allele-specific probe.

Mutations from potentially susceptible patients falling outside the coding region of inhibin can be detected by examining the non-coding regions, such as introns and regulatory sequences near or within the inhibin gene. An early indication that mutations in noncoding regions are important may come from Northern blot experiments that reveal messenger RNA molecules of abnormal size or abundance in POF patients as compared to control individuals.

Alteration of inhibin mRNA expression can be detected by any techniques known in the art. These include Northern blot analysis, PCR amplification and RNase protection. Diminished mRNA expression indicates an alteration of the wild-type inhibin gene. Alteration of wild-type inhibin genes can also be detected by screening for alteration of wild-type inhibin protein. For example, monoclonal antibodies immunoreactive with wild-type inhibin can be used to screen a tissue with lack of bound antigen indicating an inhibin mutation.

Monoclonal antibodies with affinities of 10^{-8} M^{-1} or preferably 10^{-9} to 10^{-10} M^{-1} or stronger will typically be made by standard procedures as described, eg. in Harlow & Lane, 1988 or Goding, 1986. Briefly, appropriate animals will be selected and the desired immunization protocol followed. After the appropriate period of time, the spleens of such animals are excised and individual spleen cells fused, typically, to immortalised myeloma cells under appropriate selection conditions. Thereafter, the cells are clonally separated and the supernatants of each clone tested for their production of an appropriate antibody specific for the desired region of the antigen.

Other suitable techniques for preparing antibodies involve *in vitro* exposure of lymphocytes to the antigenic polypeptides, or alternatively, to selection of libraries of antibodies in phage or similar vectors. See Huse *et al.*, 1989.

Also, recombinant immunoglobulins may be produced using procedures known in the art (see, for example, US Patent 4,816,567).

The antibodies may be used with or without modification. Frequently, antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in the literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. Patents teaching the use of such labels include US Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Antibodies specific for products of mutant alleles could also be used to detect mutant inhibin gene product. Such antibodies can be produced in equivalent fashion to the antibodies for wild-type inhibin as described above.

The immunological assay in which the antibodies are employed can involve any convenient format known in the art. Such formats include Western blots, immunohistochemical assays and ELISA assays. In addition, functional assays such as protein binding determinations, can also be used.

In summary, any approach to detecting an alteration in the underlying DNA coding for wild-type inhibin expression can be employed, whether the analysis be of the DNA itself, mRNA transcribed from the DNA or the protein which is the ultimate expression product of the DNA.

The following experimental sections outline various analyses undertaken in detail. These are included for reasons of exemplification only.

EXPERIMENTAL

Materials and Methods

POF is clinically defined by at least 4 months of amenorrhoea and two serum FSH values greater than 40 IU/L obtained one month apart before the age of 40 years. All patients were given a complete hormonal assessment to confirm their amenorrhoea was a result of POF. Elevated gonadotrophins (FSH and LH) at the onset of menstrual disturbances, indicate the likelihood of POF. POF also is associated with abnormal thyroid function and can occur as a result of an autoimmune polyglandular syndrome. The measuring of thyroxine (T4), thyroid-stimulating hormone (TSH) and in some cases tri-iodothyronine (T3) examined thyroid function.

The anti-thyroid antibodies thyroglobulin HA and microsomal HA were measured in some cases to test for the presence of an autoimmune syndrome. A karyotype was required to screen for Turner's syndrome or Turner's mosaics, or any other X chromosome abnormalities. A routine cytogenetic test was performed to identify other structural abnormalities. Fragile X testing was carried out at the Molecular Genetic laboratory at Auckland Hospital.

A total of 15 women were analysed and clinical information obtained for each woman. Two abnormal karyotypes were discovered in the 15 patients studied. One patient had a 2q33:15q13 translocation and another had a low level of Turner's mosaics. A normal number of repeats was demonstrated in all of the patients screened for fragile X premutations. We detected two variants using SSCP analysis: one in the inhibin alpha gene in patient FB1 and one in the inhibin beta A gene in patient SP7.

The patient FB1 had her first menstrual period at 14 years, and had relatively normal menstrual periods until 16 years when they stopped abruptly. At 18 years she was prescribed hormone replacement therapy. During puberty she had glandular fever but has otherwise been healthy, with no special needs.

MUTATIONAL ANALYSIS

DNA Extraction

Control DNA

Control DNA was genomic DNA extracted from 10ml samples of EDTA blood supplied by volunteers in the laboratory. Lymphocytes where isolated from blood samples using the NYCOMED Lymphoprep™ Kit. Cells were incubated at 60°C for 1 hour with 3.5ml 6M $GuHCl_2$, 250µl 7.5M NH_4Ac, 50µl 10mgml^{-1} Proteinase K and 250µl 20% Na Sarcosyl. Cell suspension was added to 2ml of cold $CHCL_3$ and then spun at 2000rpm for 3 minutes. The bottom layer was collected and added to 10ml of cold absolute ethanol to precipitate the DNA. DNA was stored in TE buffer at 2-8°C.

Patient DNA

Patient DNA: 10 ml of EDTA blood was collected by a laboratory of the patient's choice and sent to the Molecular Genetic laboratory at Auckland Hospital. DNA was

extracted at this laboratory and after a fragile X test was performed the DNA was sent to our laboratory.

Population Sample

Normal DNA samples, required for examining the prevalence of a variant in the general population, were obtained from the saliva of volunteers with signed consent. The protocol for DNA extraction from saliva was adapted from the Promega Genomic DNA Purification Kit instruction. Saliva samples were collected from subjects 10 minutes after rinsing their mouth with water to remove any food deposits. Samples were then frozen or stored at 4°C until DNA extraction could be performed. 1ml of saliva was added to 4ml of phosphate-buffered saline (PBS), pH 7.1, and centrifuged at 3000 rpm for 5 minutes. The pellet was resuspended in 180µl of PBS and 20µl of 20mg/ml RNAse A solution. Cells were lysed with 300µl of Nuclear Lysis Solution (Promega Kit) and protein was then precipitated by vortexing with 100ml of Protein Precipitation Solution (Promega Kit) followed by centrifugation at 13000 rpm for 3 minutes. The supernatant was collected and DNA precipitated with 300µl of isopropanol and centrifugation for 1 minute at 13000 rpm. The DNA pellet was resuspended overnight in 100µl of DNA Hydration Solution (Promega Kit) and stored at 2-8°C.

Polymerase Chain Reaction (PCR)

Designing and Preparing Primers

The Genbank reports for Inhibin Alpha (Mayo, Cerelli et al. 1986), Inhibin Beta A (Mason, Niall et al. 1986), and Inhibin Beta B (Mason, Niall et al. 1986), were obtained using Netscape Navigator. The DNA sequences in these reports were imported into the Edit Seq. program of the DNA Star package from Lasergene, 1994, and saved as ASCII sequence files. The Primer Select program in this package was used to design primers spanning the functional subunit of each gene.

FRAGMENT	SIZE	PRIMERS	LOCATION
Inhibin Alpha			
INHα(601bp)	601bp	For GCTGCTGCGCTGTCCCCTCTGTA	732...754
		Rev TATTTCCCAACTCTGCCTTTCCTC	1332...1309
INHα1	243bp	For GGCCCACACTCGGACCAGAC	792...811
		Rev AGCCCACAACCACCATGACAGTAG	1034...1011
INHα2	139bp	For GCTGGGCTGGGAACGGTGGAT	963...983
		Rev GGAGTAGGGCTGGGCTGGGGTAGG	1101...1078
INHα3	254bp	For CTACCCCAGCCCAGCCCTACTCCT	1079...1102
		Rev TATTTCCCCAACTCTGCCTTTCCTC	1332...1309
Inhibin Beta A			
INHβA(529bp)	529bp	For CTGGGCAAGAAGAAGAAGAAAGAA	1005...1028
		Rev CCTGGGCTGGGCAACTC	1533...1517
INHβA1	302bp	For	1071...1094
		GCAGGAGCAGATGAGGAAAAGGGAG	1372...1253
		Rev CGCATGCGGTAGTGGTTGAT	
INHβA2	268bp	For GGCACGTCCGGGTCCTCACTG	1314...1334
		Rev TCTTCATTTTGCCACTGTCTTCTC	1581...1558
Inhibin Beta B			
INHβB(586bp)	586bp	For CGTGGTGCCGGTGTTCGTGGAC	617...638
		Rev CTCCACAGCCCAACAGAATGACT	1185...1163
INHβB1	202bp	For CGTGGTGCCGGTGTTCGTGGAC	617...638
		Rev GCCGGTGGGTGCTATGAT	818...801
INHβB2	218bp	For GCACCCACCGGCTACTACG	807...825
		Rev TCCCGCTTGACGATGTTGT	1024...1006
INHβB3	241bp	For AACTCCTGCTGCATTCCCACCAA	945...967
		Rev CTCCACAGCCCAACAGAATGACT	1185...1163

Table: Primers (forward and reverse) flanking each fragment in the three inhibin subunit genes. Size of PCR products generated by each set of primers is indicated. Location of fragments is described with reference to corresponding nucleotides in the original gene.

The primers were ordered from the Life Technologies. The primers, received as dried pellets, were dissolved in sterile water to give a final concentration of 20μmol/ml.

PCR Conditions

PCR was carried out using Qiagen *Taq* polymerase and PCR buffer. Genomic DNA (1µg) was amplified in a 25µl volume reaction containing 2.5µl of PCR buffer, 25nmol of each dNTP, 5nmol of forward and reverse primers, and 0.125µl *Taq* polymerase. β-globin was used as positive control and a no DNA reaction for each primer pair acted as a negative control for all PCR reactions. Standard PCR conditions were 94°C melting for 1 minute, 58°C annealing for 1 minute and 72°C extension for 1 minute for 30 cycles. Touchdown PCR conditions were 20 cycles melting at 94°C for 45 seconds, annealing at 65°C - 55°C (-0.5°C/cycle) for 45 seconds and extension at 72°C for 1 minute, followed by 15 additional cycles with annealing at 55°C. Amplification was performed with a MT Research PT-100 thermocycler. All four Inhibin Alpha fragments INHα(601bp), INHα1, INHα2, and INHα3 along with the large Inhibin Beta A fragment, INHβA(529bp), and large Inhibin Beta B fragment, INHβB(586bp), were amplified using touchdown PCR and 5µl of Qiagen Q solution. The other two smaller Inhibin Beta A fragments (INHβA1, INHβA2) and three smaller Inhibin Beta B fragments (INHβB1, INHβB2, INHβB3, were amplified under standard PCR conditions. To ensure that a single band of expected size was present after amplification, electrophoresis of 5µl of each PCR product was carried out on a 1.5% agarose gel and visualised under UV light using an ethidium bromide stain.

Single Stranded Conformational Polymorphism (SSCP)

Preparing Samples

PCR products were diluted 1/10 with sterile water. Equal volumes of diluted sample and 2x formamide loading buffer were heated to 95°C for 3 minutes to denature samples, and immediately placed on ice to prevent DNA strands from reannealing. Electrophoresis of prepared samples (3µl) was carried out alongside non-denatured and denatured controls.

Electrophoresis

Gels consisted of 1x TBE buffer, 8% or 10% polyacylamide and no glycerol or 5% glycerol. Setting agents where 15µl 25% (w/v) Ammonium Persulphate (AMPS) and 15µl TEMED for every 10ml of non-denaturing gel. Electrophoresis was performed at room temperature (20-24°C) using 0.5% TBE buffer as the running buffer. Mini gels were run for 2-3 hours at 170V and large gels where run overnight at 200-300V.

Each fragment was optimised to ensure clear resolution of bands with maximum separation. However, variant bands may have different optimal conditions and

therefore all patient samples for each fragment were run on both large and mini gel containing 8% and 10% polyacryalmide, with (5%) and without glycerol. The final conditions for each fragment were:

Large	8% polyacrylamide gel,	5% glycerol:	INHα1, INHα3, INHβA1
Large	10% polyacrylamide gel,	5% glycerol:	INHβA2
Mini gel	8% polyacrylamide gel,	no glycerol:	INHα3, INHβB2, INHβB3
Mini gel	10% polyacryamide gel,	no glycerol:	INHα2, INHβB1

Silver Staining

Two silver staining protocols were used, one for the mini gels and one for the large gels. The mini gel protocol was faster and therefore preferred. However it used an oxidant containing $K_2Cr_2O_7$ that was too difficult to be completely removed from the large gels as they were attached to a glass plate for easy manipulation causing a problem with excessive background. Therefore a second protocol was used as an alternative for the large gels.

Mini Gel Staining

Mini gels were fixed in a mixture of 40% ethanol and 10% acetic acid for a least 30 minutes followed by two 15-minute washes in a second mixture of 10% ethanol and 5% acetic acid. Fixation was proceeded by a 15-minute wash in a $K_2Cr_2O_7$ based oxidiser. Gels were then washed in distilled water until yellow coloration of the oxidiser was completely removed. The gels were then stained in 200mls of silver reagent containing 4.08g of $AgNO_3$ for 20 minutes. A 600ml solution of developer was prepared from 17.3g of sodium carbonate, 0.3µl of formaldehyde and distilled water. Development of the gels was performed by washing in distilled water for 1 minute followed by three washes in 200ml aliquots of developer and stopped with a 5 minute wash in 5% acetic acid. The gels were washed to remove the acetic acid, transferred to Whatman 3mm filter paper, dried and stored.

Large Gel Staining

The protocol for staining the large gels differed from that of the mini gels by the fact that it did not contain an oxidiser. The large gels, which were attached to a large glass with 250µl of silane gel binding solution, were fixed in a 10% acetic acid solution for 1-2 hours followed by 2-3 washed in distilled water to remove excess acetic acid. Gels were then placed in a silver staining solution consisting of 3g of silver nitrate and 4.5ml of formaldehyde in 3L of distilled water for 2 hours. Four

litres of developer containing 120g of sodium carbonate was chilled at -80°C for 30 minutes along with 2L of distilled water and the 10% acetic acid stop solution. Immediately prior to use, the developer had 6.5ml of formaldehyde and 800µl of sodium thiosulphate (10mg/ml) added. The gel was rinsed in the chilled water for 5 seconds and then placed in the 1st half of the developer until the bands began to appear. It was then transferred to the 2nd half of the developer until the bands were clear and then immediately stopped in the chilled acetic acid for 5 minutes. The acetic acid was then washed out and the gel was placed into a 0.5M NaOH solution to remove the gel from the glass plate. The gel was transferred to Whatman 3mm filter paper, dried and stored.

DNA Sequencing

The large PCR fragments INHα(601bp), INHβA(529bp) and INHβB(586bp), that spanned the entire functional region of each of the three inhibin genes, were used as template for DNA sequencing. PCR products were purified with Promega's Wizard PCR Product Purification Kit to remove salts and any leftover enzyme or primers from the amplification reaction. The Sequencing Faculty in the Biological Sciences Department sequenced the PCR products at the University of Auckland. Sequencing was performed on the 373 model automated sequencer using 2µl of template DNA.

Characterisation of Variant

The PCR products from a sample of the population were analysed for the variant G^{913} to A^{913} in exon 2 of the Inhibin Alpha subunit gene. RFLP using Bst71I as the restriction enzyme was used to determine if this variant was a normal polymorphism or a mutation possibly responsible for POF. Normal DNA was collected from saliva samples of 48 men and women of all ages as a representation of the general population. DNA from the lab, with a known DNA sequence, acted as wildtype DNA and DNA from the patient FB1 acted as a control for the variant. DNA extraction and PCR amplification of the INHα1 fragment was carried out as previously described. The restriction enzyme digest was conducted in 1x restriction buffer using 2.5U of Bst71I, 0.2µl of acetylated BSA, 5µl of PCR product and sterile water to give a total reaction volume of 20µl. The reaction mixtures were incubated at 37°C for 1-2 hours, electrophoresed on a 2% agarose gel and stained with ethidium bromide. To allow better separation of the fragments some of the samples were also electrophoresised on an 8% polyacrylamide gel which were stained with Syber Gold. The uncut DNA control was also incubated in a reaction mixture containing all the above reagents except the Bst71I enzyme and run along side the restricted PCR products. The

child bearing). For non-carriers, in a family with a history of POF testing will bring peace of mind and will remove the need for surveillance.

The identification of inhibin as a putative POF susceptibility gene has implications beyond early detection. The possibility of preventative approaches to delay the onset of POF is also raised.

These involve, primarily, the direct administration to the susceptible female of wild-type inhibin in a sufficient amount to restore the active concentration of inhibin to putatively "normal" levels.

For such an approach, the inhibin can be prepared as a medicament, in combination with conventional carriers, vehicles, diluents or excipients.

There is also the possibility of a curative or corrective approach using gene therapy. This will involve supplying wild-type inhibin function to an individual who carries mutant inhibin genes. Supplying such a function should prevent POF developing. The wild-type inhibin gene or a part of the gene may be introduced into cells within such an individual in a vector such that the gene remains extrachromosomal. In such a situation, the gene will be expressed by the cell from the extrachromosomal location. More usual is the situation where the wild-type inhibin gene or a part thereof is introduced into the mutant cell in such a way that it integrates into the genomic DNA. Less usual is the situation where the wild-type inhibin gene or a part thereof is introduced into the mutant cell in such a way that it recombines with the endogenous mutant inhibin gene present in the cell. Such recombination requires a double recombination event which results in the correction of the inhibin gene mutation. Vectors for introduction of genes both for recombination and for extrachromosomal maintenance are known in the art, and any suitable vector may be used. Methods for introducing DNA into cells such as electroporation, calcium phosphate co-precipitation and viral transduction are known in the art.

As generally discussed above, the wild-type inhibin gene or fragment, where applicable, may be employed in gene therapy methods in order to increase the amount of the expression products of such genes in target cells. Such gene therapy is particularly appropriate for use in cells in which the level of inhibin polypeptide is absent or diminished compared to normal cells. It may also be useful to increase the level of expression of a given inhibin gene even in those cells in which the mutant gene is expressed at a "normal" level, but the gene product is not fully functional.

Silver Stain Oxidising Solution

5g	$K_2Cr_2O_7$ 0.034M
1.43ml	50% Nitric Acid

Add H_2O to 500ml and mix thoroughly. Store at 4°C.

50x TAE buffer

242g	Tris Base
57.1ml	Glacial Acetic Acid
37.2g	$Na_2EDTA.2H_2O$ (2mM)

Make up to 1 litre with H_2O and mix well. Store at room temperature.

10x TBE buffer

108g	Tris Base	890mM
55g	Boric Acid	890mM
40ml	0.5M EDTA Solution (pH8)	20mM

Make up to 1 litre with H_2O. Store at room temperature.

TE buffer

10ml	1M Tris-Cl (pH 7.4)	10.0mM
2ml	0.5M EDTA (pH5)	1mM

Make up to 1 liter with H_2O. Autoclave and store at room temperature.

Results

Mutation Detection

Two variants were detected using SSCP analysis. The INHα1 fragment displayed two extra bands in the patient FB1 compared against the wildtype (Lab DNA)). This variant could only be detected on the large gels where the bands were adequately separated emphasising the need to screen for mutations with more than one set of conditions. The rest of the patient samples for this fragment were the same as the wildtype DNA. The other variant was seen in the INHβA1 fragment of the sporadic patient SP7.

DNA sequencing

The fragments INHα(601bp), INHβA(529bp), and INHβB(586bp) from every patient DNA sample were amplified, including the lab DNA. Samples were purified before sequencing to remove any leftover reagents and primers from the PCR reaction. Sequencing was performed on all samples to confirm the results of the SSCP analysis

and to ensure no variants had been missed. No additional variants were discovered using DNA sequencing that had not been indicated using SSCP analysis.

DNA sequencing confirmed the INHα1 variant detected in the patient FB1 using SSCP. The variant was the result of a G→A missense substitution at nucleotide 913 which alters codon 196 from GCT to ACT, resulting in an alanine to threonine amino acid substitution in the functional domain of the inhibin alpha subunit gene. To confirm that the variant was not a sequencing error the INHα1 fragment was amplified from the original DNA sample FB1 and sequenced in both directions. Again the same sequence variation was identified.

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RFLP analysis did not identify the presence of the G^{913} to A^{913} variant in any of the 48 normal DNA samples studied. Uncut PCR products electrophoresised on a 1.5% agarose gel revealed the normal 244bp fragment. Among the 96 alleles digested with Bst71I all demonstrated the wildtype banding pattern of the 85bp and 134bp fragments. The variant demonstrated heterozygousity, yielding fragments of 85bp and 159bp and also the 134bp fragment, characteristic of the wildtype. The 25bp fragment was undetectable in all the samples, as it was too small to be seen on the agarose gels and ran off the bottom of the polyacrylamide gels.

Discussion

The INHα G913A transition resulted in an alanine to threonine substitution at codon 196. RFLP analysis revealed that this variant is rare in the normal population. All ninety six alleles screened were negative for this variant. It is likely to be a non-conservative substitution as the alanine to threonine results in the addition of an

aliphatic hydroxyl group in the side chain of the functional group. The hydroxyl group results in threonine being more hydrophilic and reactive than alanine.

The human inhibin alpha gene demonstrates 80% homology with equine, bovine, porcine, ovine, rat and mouse sequences (Yamanouchi, Yoshida et al. 1995). The amino acid alanine was conserved at the site of the mutation in all of these sequences, with exception to the rat sequence. The rat sequence contained a serine instead of an alanine. Serine is the hydroxylated version of alanine. Threonine, however, has the addition of a methyl group as well as the hydroxyl group making it a large amino acid. Therefore, the substitution of alanine for serine in the rat is a more conservative alteration than the threonine substitution induced by the INHα mutation. Also, the rat is most divergent when compared with the human INHα gene of all these species.

Inhibin alpha shows amino acid sequence homology to TGF-β2 (transforming growth factor-β2) and OP-1 (oestrogenic protein-1) of 24% and 26%, respectively (Kinsley, 1994). The 3-dimensional crystal structure of TGF-β2 and OP-1 is known, and all three appear to show a characteristic 7-cystein domain resulting in a ring structure named the cysteine knot. The G193A transition is adjacent to the first cysteine residue that forms a disulphide bond with the fifth cysteine residue. Consequently, the mutation is draw into close proximity with the fourth cysteine residue, which forms the disulphide bond involved in dimerisation. Therefore, this mutation has the potential to disrupt the binding of the inhibin alpha subunit to the inhibin beta subunit. This may be directly, by disrupting the disulphide bond involved in dimer formation, or indirectly, by disrupting the tertiary structure of the cysteine knot and therefore inhibiting its ability to dimerise.

INDUSTRIAL APPLICATION

The demonstration that mutations in inhibin genes (particularly INHα) are associated with instances of POF has a number of implications. As indicated above, the primary implication is in a method of detection of a risk of a predisposition to POF or a method of POF diagnosis.

Early at-risk determination provides the opportunity for early intervention. Carriers of the mutation could choose to have treatment prior to the emergence of any problem. Testing also enables carriers to make important life decisions (eg. early

adenovirus vector. The trimolecular complex is then used to infect cells. The adenovirus vector permits efficient binding, internalization, and degradation of the endosome before the coupled DNA is damaged.

Liposome/DNA complexes have been shown to be capable of mediating direct *in vivo* gene transfer. While in standard liposome preparations the gene transfer process is nonspecific, localized *in vivo* uptake and expression have been reported in tissue deposits, for example, following direct *in situ* administration (Nabel, 1992).

Those persons skilled in the art will appreciate that the above description is provided by way of example only and that it is limited only by the lawful scope of the appended claims.

Gene therapy would be carried out according to generally accepted methods, for example as described by Kren *et al.*, (1998), or as described by Friedman in *Therapy for Genetic Disease*, T. Friedman, ed., Oxford University Press (1991), pp 105-121. Cells from a patient would be first analyzed by the methods described above, to ascertain the production of inhibin polypeptide. A virus or plasmid vector, containing a copy of the inhibin gene linked to expression control elements and capable of replicating inside the target cells, is prepared. Suitable vectors are known, such as disclosed in US Patent 5,252,479 and PCT published application WO 93/07282. The vector is then injected into the patient, either locally at the site of the target cells or systemically (in order to reach any target cells that may be at remote sites). If the transfected gene is not permanently incorporated into the genome of each of the targeted cells, the treatment may have to be repeated periodically.

Gene transfer systems known in the art may be useful in the practice of the gene therapy methods. These include viral and nonviral transfer methods. A number of viruses have been used as gene transfer vectors, including papovaviruses (eg. SV40, Madzak *et al.*, (1992)), adenovirus (Berkner (1992)), vaccinia virus (Moss (1992)), adeno-associated virus (Muzyczka (1992)), herpesviruses including HSV and EBV (Margolskee (1992); Johnson *et al.*, (1992); Fink *et al.*, (1992); Breakfield and Geller, (1987); Freese *et al.*, (1990)), and retroviruses of avian (Petropoulos *et al.*, (1992), murine (Miller (1992)); and human origin (Shimada *et al.*, (1991); Helseth *et al.*, (1990); Page *et al.*, (1990); Buchschacher and Panganiban (1992)).

Nonviral gene transfer methods known in the art include chemical techniques such as calcium phosphate coprecipitation (Pellicer *et al.*, (1980)); mechanical techniques, for example microinjection (Anderson *et al.*, (1980)); membrane fusion-mediated transfer via liposomes (Lim *et al.*, (1992)); and direct DNA uptake and receptor-mediated DNA transfer (Wolff *et al.*, (1990); Wu *et al.*, (1991)). Viral-mediated gene transfer can be combined with direct *in vivo* gene transfer using liposome delivery, allowing one to direct the viral vectors to the target cells. Alternatively, the retroviral vector producer cell line can be injected into the patient (Culver *et al.*, 1992). Injection of producer cells would then provide a continuous source of vector particles.

In an approach which combines biological and physical gene transfer methods, plasmid DNA of any size is combined with a polylysine-conjugated antibody specific to the adenovirus hexon protein, and the resulting complex is bound to an

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wildtype INHα1 PCR product when digested with Bst71I generates three separate bands of 85bp, 25bp and 134bp. In the presence of the G^{913} to A^{913} variant at codon 196, the enzyme recognition site $CGTCG(n)_{12}$ is abolished generating only two bands of 85bp and 159bp. A heterozygous sample will display all four fragments.

Buffers and Solutions

Agarose gels

100µl	1xTAE buffer
8µl	Ethidium bromide
1.0 g	agarose (1.0 %)

Ethidium Bromide Solution (10mg/ml)

0.2g	Ethidium Bromide
20ml	H_2O

Mix well and store in dark or in a foil-wrapped bottle.

Formamide Loading buffer (2x)

0.50% (w/v)	Bromophenol Blue
0.05% (w/v)	Xylene cyanol FF
20mM	EDTA

Prepare in deionised formamide. Do not sterilise and store at -20°C.

Gel Loading Buffer (6x)

0.25% (w/v)	Bromophenol Blue
15% (w/v)	Ficoll 400

Store for use at 4°C.

Phosphate Buffered Saline (PBS)

8.0g	Sodium Chloride (137mM)
0.2g	Potassium Chloride (2.7mM)
2.16g	$Na_2HPO_4.7H_20$ (8.0mM)
0.2g	KH_2PO_4 (1.5mM)
800ml	H_2O

Add HCl to adjust to pH 7.3. Autoclave and store at room temperature.

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